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Targeted protein phosphorylaytion is a key event that serves to transduce DNA damage-induced cell signals from upstream sensors to downstream effectors. ATR kinase phosphorylates BRCA1 and Rad17 upon DNA damage. Furthermore, the phosphorylation of BRCA1 can also influence the phosphorylation of other substrates by ATR. As a first approach to evaluate the functional consequence of IR-induced site-specifically phosphorylated residues on BRCA1, we have established a BRCA1-dependent transcription-based assay which assesses the BRCA1-dependent repression function of ZBRK1 in mammalian cell from a reporter template bearing ZBRK1 DNA-binding sites. Functional dissection of ZBRK1 led to identification and characterization of a novel BRCA1-dependent repression domain encompassing ZBRK1 zinc fingers 5-8 and the unique C-terminus. This C-terminal repression domain functions in a BRCA1-dependent, histone deactylase-dependent and promoter-specific. Significantly, we also found that the BRCA1-dependent transcriptional repression domain on ZBRK1 includes elements that modulate its sequence-specific DNA-binding activity. This study revealed an unanticipated dual function for the ZBRK1 zinc fingers in DNA-binding and BRCA1-dpendent transcriptional repression. Our BRCA1-dependent ZBRK1 repression assay may now be exploited to evaluate the influence of IR-induced site-specific phosphorylation of BRCA1 on its sequence-specific co-repressor function.

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INTRODUCTION

Targeted protein phosphorylation is a primary cellular response to DNA damage and a signal event through which downstream effector molecules are mobilized into action by upstream DNA damage response sensors. BRCA1 (Breast Cancer 1), a hereditary breast- and ovarian-specific tumor suppressor, plays a central role in the cellular response to DNA damage by virtue of its dual function in DNA damage-induced cell cycle checkpoint control and DNA repair. In response to DNA damage, BRCA1 can be phosphorylated by both the ATM (Ataxia telangiecstasia mutated) and ATR (ATM and Rad3 related) kinases. RAD17 (Radiation sensitive complimentary group protein 17) is another ATR substrate and downstream DNA damage-respone effector molecule. At the molecular level, Rad17 is phosphorylated, and this phosphorylation is required for DNA damage-induced cell cycle checkpoint control during G1 phase in breast cancer cells. Over-expression of human RAD17 (hRAD17) has been linked to metastastic breast cancer. A better understanding of how DNA damage-induced BRCA1 and hRAD17 phosphorylation modulate their respective functions may reveal novel insight into the molecular events underlying breast tumorigeneis and metastasis.

BODY

In an initial effort to investigate the influence of phosphorylation on BRCA1, I have established a BRCA1-dependent transcription-based assay to evaluate the effects of IR-induced site-specifically phosphorylated residues on BRCA1. This assay is based on the functional interaction between BRCA1 and the sequence-specific transcriptional repressor protein ZBRK1 (Zinc Finger BRCA1 interacting protein with a KRAB domain 1). Previously, our lab showed that BRCA1 is a co-repressor of ZBRK1, a sequence-specific DNA-binding transcriptional repressor of the DNA damage-inducible *GADD45* gene that functions in G2/M cell cycle checkpoint control (1). In addition to *GADD45,* (Growth Arrest and DNA Damage inducible) gene potential ZBRK1 binding sites have been identified in other DNA damage-inducible genes, indicating a prospective global role for ZBRK1 and BRCA1 in the coordinate regulation of DNA damage-response genes (1). Based on these previous observations, we have proposed a model whereby ZBRK1 and BRCA1 coordinately repress a group of DNA damage response genes in the absence of genotoxic stress and, further, that DNA damage-induced cell signals relieve this repression, thereby permitting DNA damage-induced activation of these genes.

The DNA damage-induced cell signals that relieve coordinate repression of DNA damage response genes by ZBRK1 and BRCA1 is likely to involve phosphorylation. In fact, previous studies have revealed that IR-induced protein phosphorylation is required to relieve BRCA1-mediated repression of the *GADD45* (2). To facilitate studies designed to determine the effects of targeted mutations at identified sites of IR (ionizing radiation-induced) phosphorylation within BRCA1 on its ability to control transcription, we established a BRCA1-dependent ZBRK1 transcriptional repression assay. This assay is designed to assess the BRCA1-dependent repression function of ZBRK1 in mammalian cells from a reporter template bearing ZBRK1 DNA-binding sites. To establish the utility of this system as a means to study BRCA1-dependent ZBRK1

repression, we used this system as a functional readout during experiments designed to functionally dissect ZBRK1. This study was completed and published within the last year of this training period (3). In this study, we reported the identification and characterization of a novel BRCA1-dependent transcriptional repression domain within ZBRK1 composed of ZBRK1 zinc fingers 5-8 along with sequences in the unique ZBRK1 C-terminus. This C-terminal repression domain functions in a BRCA1-, histone deacetylase-, and promoter-specific manner and is thus functionally distinguishable from an N-terminal repression domain in ZBRK1, which exhibits no BRCA1 dependency and broad promoter specificity. Significantly, we also found that the BRCA1-dpendent transcriptional repression domain on ZBRK1 includes elements that modulate its sequence-specific DNA-binding activity. In addition to demonstrating the utility of this assay to quantify BRCA1-dependent ZBRK1 transcriptional repression, this study also revealed an unanticipated dual function for the ZBRK1 zinc fingers in DNA-binding and BRCA1-dpendent transcriptional repression. As such, this study sheds new light on the mechanistic basis by which BRCA1 mediates sequence-specific control of DNA damage-responsive gene transcription.

Our BRCA1-dependent ZBRK1 repression assay may now be exploited to evaluate the influence of IR-induced site-specific phosphorylation of BRCA1 on its sequence-specific co-repressor function. We plan to evaluate the influence of IR on the ability of ectopically expressed wild-type BRCA1 to function as a ZBRK1-specific co-repressor. We predict, and will test the possibility, that IR leads to phosphorylation of BRCA1 and consequent relief of ZBRK1-directed repression from the ZBRK1 target reporter plasmid through disruption of the ZBRK1/BRCA1 interaction. Subsequently, we will evaluate BRCA1 derivatives bearing site-directed mutations at sites of IR-induced phosphorylation for their respective abilities to function as IR-reversible co-repressors of ZBRK1. We predict that BRCA1 derivatives bearing mutations at identified sites of IR-induced phosphorylation will function as constitutive ZBRK1 co-repressors through an IR-insensitive interaction with ZBRK1. This assay should therefore provide us with a rapid and facile approach to test the hypothesis that IR-induced site-specific phosphorylation of BRCA1 modulates its transcriptional regulatory activities.

KEY RESEARCH ACCOMPLISHMENTS TO DATE

- Novel discovery within ZBRK1 of functionally bipartite zinc fingers with dual roles in sequence-specific DNA-binding and BRCA1-dependent transcriptional repression.

- Establishment of a BRCA1-dependent ZBRK1 transcriptional repression assay that will expedite studies designed to test the hypothesis that IR-induced site-specific phosphorylation of BRCA1 modulates its transcriptional regulatory activities.

REPORTABLE OUTCOMES TO DATE.

Manuscripts:

1. Tan, W. Zheng, L., Lee, W.-H. and Boyer, T.G. (2004). Functional dissection of Transcription Factor ZBRK1 Reveals Zinc Fingers with Dual Roles in DNA-binding and BRCA1-dependent Transcriptional Repression. J. Biol. Chem. **279**: 6576-6587.

Meeting Abstract:

1. Tan, W. Zheng, L., Chen, P.-L., Lee, W.-H., and Boyer, T.G. (2003). Molecular dissection of a BRCA1-dependent transcriptional repression domain. Mechanisms of Eukaryotic Transcription. Cold Spring Harbor, New York.

CONCLUSIONS

We have succeeded establishing an efficient and reliable BRCA1-dependent transcription-based functional assay which has permitted us to make the additional novel discovery that ZBRK1, a BRCA1-dependent transcriptional repressor, harbors zinc fingers with dual roles in sequence-specific DNA-binding and BRCA1-dependent transcriptional repression. This finding sheds new light on the mechanistic basis by which BRCA1 mediates sequence-specific control of DNA damage-responsive gene transcription. Our current efforts are targeted at identifying DNA damage-induced site-specific phosphorylation events with potential functional relevance to the role of BRCA1 in transcription. Hence, we will exploit these BRCA1-dependent transcription-based assays to analyze of the effects of targeted BRCA1 mutations at identified sites of phosphorylation on its transcriptional regulatory activities. These studies should illuminate further the molecular basis for the caretaker properties of BRCA1.

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Functional Dissection of Transcription Factor ZBRK1 Reveals Zinc Fingers with Dual Roles in DNA-binding and BRCA1-dependent Transcriptional Repression*

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The breast- and ovarian-specific tumor suppressor BRCA1 has been implicated in both activation and repression of gene transcription by virtue of its direct interaction with sequence-specific DNA-binding transcription factors. However, the mechanistic basis by which BRCA1 mediates the transcriptional activity of these regulatory proteins remains largely unknown. To clarify this issue, we have examined the functional interaction between BRCA1 and ZBRK1, a BRCA1-dependent KRAB eight zinc finger transcriptional repressor. We report here the identification and molecular characterization of a portable BRCA1-dependent transcriptional repression domain within ZBRK1 composed of zinc fingers 5–8 along with sequences in the unique ZBRK1 C terminus. This C-terminal repression domain functions in a BRCA1-, histone deacetylase-, and promoter-specific manner and is thus functionally distinguishable from the N-terminal KRAB repression domain in ZBRK1, which exhibits no BRCA1 dependence and broad promoter specificity. Significantly, we also find that the BRCA1-dependent transcriptional repression domain on ZBRK1 includes elements that modulate its sequence-specific DNA binding activity. These findings thus reveal the presence within ZBRK1 of functionally bipartite zinc fingers with dual roles in sequence-specific DNA-binding and BRCA1-dependent transcriptional repression. We discuss the implications of these findings for the role of BRCA1 as ZBRK1 co-repressor.

Germ line inactivation of the gene encoding BRCA1 confers a cumulative lifetime risk of female breast and ovarian cancer (1–3). Although the mechanistic basis for its tissue- and gender-specific tumor suppressor activity remains poorly defined, BRCA1 nonetheless fulfills a broad function in the maintenance of global genome stability (4–10). The underlying basis for this caretaker activity likely derives from the role of BRCA1 as a conduit in the cellular DNA damage response, wherein it serves to couple DNA damage-induced signals to downstream responses including DNA damage repair and cell cycle checkpoint activation (6, 7, 11–21). Several potentially overlapping cellular activities have been ascribed to BRCA1, each of which could underlie its ability to control signal output. For example, BRCA1 has been implicated in chromatin remodeling, ubiquitinylation, recombination, and transcriptional regulation (6, 22–28). The extent to which these pleiotropic activities contribute to the caretaker function of BRCA1 is presently unknown; however, the fact that each of these BRCA1-associated activities are similarly abrogated by cancer-predisposing BRCA1 missense mutations suggests a strong correlative link between their discharge and BRCA1-mediated tumor suppression.

With respect to its role in transcription control, BRCA1 has been implicated in both activation and repression of genes linked to a variety of biological processes, including cell growth control and DNA replication and repair (21, 29–31). Thus, by virtue of its transcriptional regulatory activity, BRCA1 could influence cellular responses downstream of DNA damage signals, and this activity could contribute to its caretaker function.

The precise role of BRCA1 in gene-specific transcription control has yet to be definitively established. Because it exhibits no sequence-specific DNA binding activity, it seems likely that BRCA1 is targeted to specific genes through its functional interaction with sequence-specific DNA-binding transcription factors. Direct evidence to support this hypothesis has come from the identification of multiple DNA-binding transcription factors with which BRCA1 has been shown to physically interact and functionally synergize, including p53, c-Myc, estrogen receptor α, androgen receptor, OCT-1, NF-YA, and ZBRK1 (32–40). However, the underlying mechanism by which BRCA1 mediates the transcriptional stimulatory or repressive effects of these regulatory proteins has not been established.

We have been studying the functional interaction between BRCA1 and the transcriptional repressor ZBRK1 as a model system to understand the mechanistic basis by which BRCA1 mediates sequence-specific transcription control. Initially identified by virtue of its physical interaction with BRCA1, ZBRK1 (Zinc finger and BRCA1-interacting protein with a KRAB domain 1) is a member of the Kruppel-associated box-zinc finger protein (KRAB-ZFP) family of transcriptional repressors (39, 41). Typically, KRAB-ZFPs bind to their corresponding target genes through tandem C-terminal C_2H_2 zinc fingers and repress transcription through an N-terminal KRAB domain, which silences gene expression through the indirect recruitment of histone deacetylases, histone methyltransferases, and heterochromatin proteins (41–47). Like other KRAB-ZFPs, ZBRK1 harbors an N-terminal KRAB domain. However, ZBRK1 is atypical among KRAB-ZFPs due to the fact that it harbors 8 central C_2H_2 zinc fingers and a unique C terminus

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that is absent among the larger family of KRAB-ZFPs.

Through its 8 central zinc fingers, ZBRK1 binds to a compositionally flexible 15-bp DNA sequence, GGG*xxx*CAG*xxx*TTT (where *x* is any nucleotide) (39). A search for potential ZBRK1 DNA-binding sites in existing genes led to intron 3 of GADD45a, a functionally important DNA damage-response effector known to be regulated transcriptionally by BRCA1 (21, 32, 39). Functional analysis revealed that ZBRK1 represses *GADD45a* gene transcription through its intron 3 DNA-binding site in a BRCA1-dependent manner, thus revealing BRCA1 to be a ZBRK1 co-repressor (39). Significantly, familial breast cancer-derived mutants of BRCA1 that disrupt its interaction with ZBRK1 abrogate its co-repressor activity, suggesting that its co-repressor function may be important for the tumor suppressor properties of BRCA1 (39). The regulation of *GADD45a* gene transcription is likely to be complex and controlled coordinately by ZBRK1 and BRCA1 in concert with other trans-acting factors, including p53, OCT1, and NF-YA, that function through cis-acting sequences present in the *GADD45a* promoter and intron 3 regions (21, 32, 39, 48, 49).

In addition to *GADD45a*, potential ZBRK1-binding sites have been identified in other DNA damage-response genes that are also regulated by BRCA1, including p21, Bax, and GADD153 (39). This observation suggests a potentially broader role for BRCA1 and ZBRK1 in the coordinate transcriptional regulation of diverse DNA damage-response genes. To begin to explore the mechanism by which BRCA1 mediates sequence-specific transcriptional repression through ZBRK1, we have pursued in greater depth the physical and functional interaction between these two proteins. We report here the identification and molecular characterization of a portable BRCA1-dependent transcriptional repression domain within ZBRK1 composed of zinc fingers 5–8 along with sequences in the unique ZBRK1 C terminus. This C-terminal repression domain functions in a BRCA1-, histone deacetylase (HDAC)-,[1] and promoter-specific manner and is thus functionally distinguishable from the N-terminal KRAB repression domain in ZBRK1, which exhibits no BRCA1 dependence and broad promoter specificity. Significantly, we also find that the BRCA1-dependent C-terminal transcriptional repression domain within ZBRK1 is composed of elements that modulate sequence-specific DNA-binding by zinc fingers 1–4. These findings thus reveal an unanticipated dual function for the ZBRK1 zinc fingers in DNA binding and transcriptional repression, and further shed new light on the mechanistic role of BRCA1 in sequence-specific transcriptional control.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis

Expression Plasmids—pMAL-C2-TEV-ZBRK1 ΔK for expressing MBP-ZBRK1 ΔK in *Escherichia coli* was derived from pGEPK3-ZBRK1 ΔK, which is a derivative of pCNF-ZBRK1 ΔK (39). Briefly, a BamHI-XhoI fragment carrying ZBRK1 cDNA sequences encoding ZBRK1 amino acids 144–532 (lacking the N-terminal KRAB domain) was subcloned into the BamHI and XhoI sites of pMAL-C2-TEV (provided by Dr. P. Renee Yew), thereby generating a translational fusion of MBP and ZBRK1 ΔK. C-terminal truncation mutants of MBP-ZBRK1 ΔK bearing stepwise deletions of individual ZBRK1 zinc fingers were expressed from pMAL-C2-TEV-ZBRK1 ΔK deletion derivatives, each of which was constructed by PCR-based subcloning. Briefly, sequences encoding ZBRK1 ΔK within pMAL-C2-TEV-ZBRK1 ΔK were liberated as a BamHI-HindIII fragment and replaced with corresponding PCR-generated ZBRK1 deletion fragments using a common upstream primer corresponding to MBP sequences and unique downstream primers within the C terminus of each zinc finger (defined here as the seventh amino acid residue C-terminal to the last histidine residue of each C_2H_2 zinc finger). Individual MBP-ZBRK1 ΔK deletion derivatives encode the following ZBRK1 amino acids (aa): MBP-ZBRK1 ΔK 8ZF (aa 144–431); 7ZF (aa 144–403); 6ZF (aa 144–377); 5ZF (aa 144–347); 4ZF (aa 144–319); 3ZF (aa 144–291); 2ZF (aa 144–263); and 1ZF (aa 144–235). Individual MBP-ZBRK1 ΔK broken finger (BF) mutants were generated by PCR-based site-directed mutagenesis of pMAL-C2-TEV-ZBRK1 ΔK using the QuickChange II site-directed mutagenesis kit following the manufacturer's recommendations (Stratagene, La Jolla, CA). Each broken finger mutant bears a histidine (CAT codon) to asparagine (aaT codon) substitution mutation at the first of the two conserved histidine residues within the targeted C_2H_2 zinc finger.

ZBRK1 5ZFC was expressed as a GAL4 DNA-binding domain fusion in mammalian cells from the plasmid GAL4-ZBRK1 5ZFC, constructed by subcloning a PCR-amplified ZBRK1 cDNA fragment encoding amino acids 319–532 (encompassing zinc finger 5 through the C terminus) into the SalI and HindIII sites of pM (Clontech, Palo Alto, CA). N- and C-terminal truncation derivatives of GAL4-ZBRK1 5ZFC bearing stepwise deletions of individual zinc fingers and C-terminal sequences, respectively, were generated by PCR-based subcloning. Briefly, the SalI-HindIII fragment within GAL4-ZBRK1 5ZFC encoding ZBRK1 5ZFC was replaced with corresponding PCR-generated ZBRK1 deletion fragments. Individual GAL4-ZBRK1 5ZFC deletion derivatives encode the following ZBRK1 amino acids (aa): GAL4-ZBRK1 6ZFC (aa 347–532); 7ZFC (aa 375–532); 8ZFC (aa 403–532); C (aa 431–532); GAL4-ZBRK1 5ZFC Δ1 (aa 319–523); 5ZFC Δ2 (aa 319–503); and 5ZFC Δ3 (aa 319–483). Broken finger derivatives of GAL4-ZBRK1 5ZFC were generated by PCR amplification of ZBRK1 sequences encoding amino acids 319–532 from individual pMAL-C2-TEV ZBRK1 ΔK BF mutants and subsequent replacement of the SalI-HindIII wild-type ZBRK1 fragment in GAL4-ZBRK1 5ZFC.

ZBRK1 5ZFC and its truncation and broken finger derivatives were expressed in yeast as GAL4 activation domain fusions using pGADT7 (Clontech, Palo Alto, CA). Briefly, ZBRK1 5ZFC and its truncation (6ZFC, 7ZFC, 8ZFC, 0ZFC, 5ZFC Δ1, 5ZFC Δ2, and 5ZFC Δ3) and broken finger (BF5, BF6, BF7, and BF8) derivatives were excised as BamHI-blunted SalI fragments from GAL4-ZBRK1 5ZFC and its corresponding derivative plasmids, and subcloned into the BamHI and blunted XhoI sites in pGADT7. A BRCA1 cDNA fragment (encoding amino acids 341–748) encompassing the ZBRK1-binding domain (39) was PCR-amplified and subcloned into the EcoRI and SalI sites of pGBKT7, thereby generating a translational fusion of the GAL4 DNA-binding domain with BRCA1 amino acids 341–748 in the plasmid pGBKT7-BRCA1.

The ZBRK1 KRAB domain was expressed as a GAL4 DNA-binding domain fusion in mammalian cells from GAL4-ZBRK1 KRAB, constructed by subcloning a PCR-amplified ZBRK1 cDNA fragment encoding amino acids 1–85 into the BamHI and blunted XbaI sites of pM2 (50). All PCR-based subcloning was performed using *Pfu* DNA polymerase (Stratagene, La Jolla, CA), and the integrity of individual deletion and substitution mutations was confirmed by DNA sequence analysis.

Reporter Plasmids—pG_5TK-Luc carrying five copies of the GAL4 DNA-binding site upstream of the herpes simplex virus thymidine kinase (TK) promoter (sequences corresponding to −105 to +51, where +1 is the transcription start site) driving expression of the gene encoding firefly luciferase was constructed by replacing a HindIII-BglII fragment from pSBS-GAL-TK-Luc (provided by Dr. Tony Ip) with a HindIII-BglII fragment from pG_5TK-CAT (provided by Dr. P. Renee Yew), thus positioning five copies of the GAL4 DNA-binding site and the TK promoter upstream of the firefly luciferase gene. pG_5SV40-Luc carrying five GAL4 DNA-binding sites upstream of the SV40 promoter driving expression of the firefly luciferase gene has been described previously (39). pG_5SNRPN-Luc (provided by Dr. Paul A. Wade) carries five GAL4 DNA-binding sites upstream of the human small nuclear ribonucleoprotein N promoter driving expression of the firefly luciferase gene (51).

Recombinant Protein Expression and Purification

MBP-ZBRK1 fusion proteins were expressed in and purified from *E. coli* strain BL21 Star (DE3) pLysS (Invitrogen). Briefly, cells were grown at 37 °C to an A_{600} of 0.6. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration 0.3 mM, and the cells were transferred to 25 °C for another 3.5 h. Cells were pelleted, washed once with phosphate-buffered saline, and then resuspended in MBP binding buffer (25 mM Tris-HCl, pH 7.5; 1 mM EDTA; 200 mM NaCl; 20 μM

[1] The abbreviations used are: HDAC, histone deacetylase; EMSA, electrophoretic mobility shift assay; MEFs, mouse embryo fibroblasts; aa, amino acids; MBP, maltose-binding protein; PMSF, phenylmethylsulfonyl fluoride; TSA, trichostatin A; ZRE, ZBRK1-response element; BF, broken finger; TK, thymidine kinase; WT, wild type; Mut., mutant; HSV, herpes simplex virus.

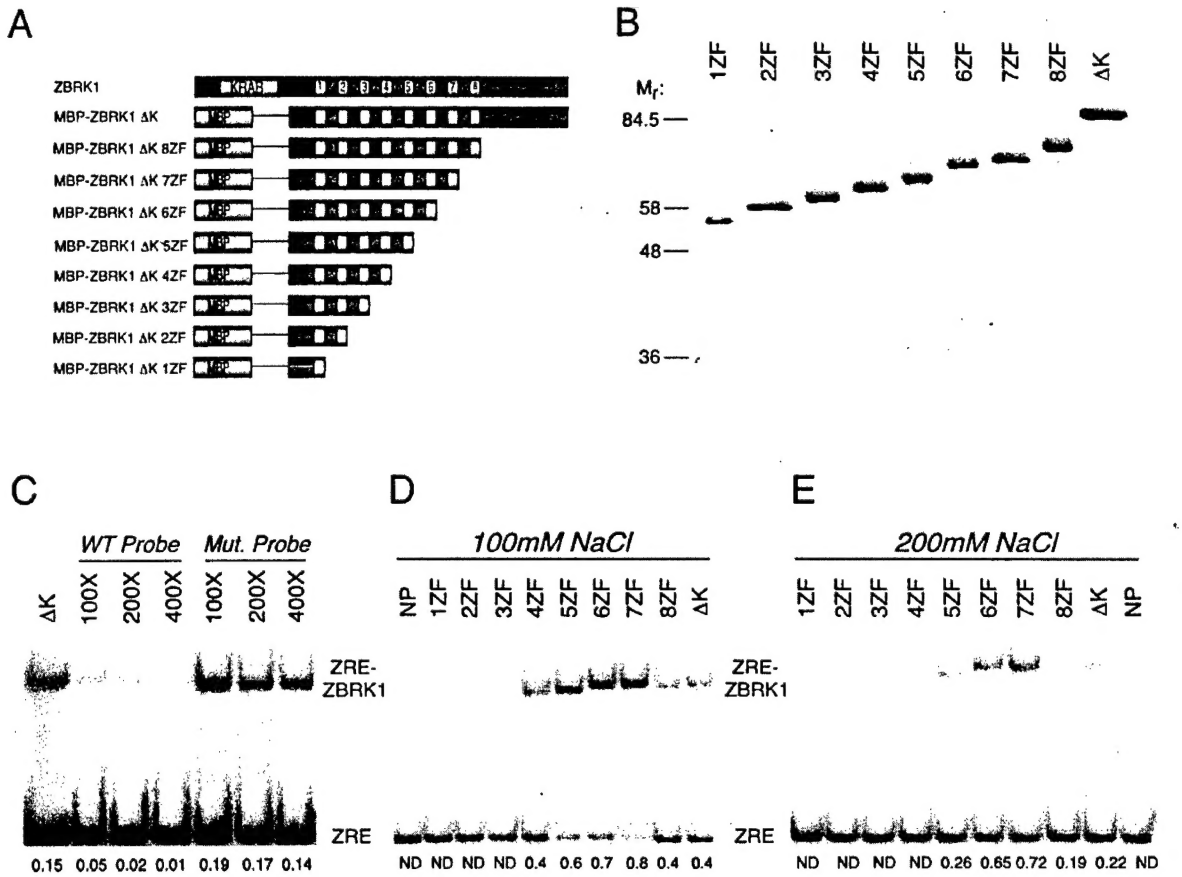

FIG. 1. **Identification of DNA binding determinants on ZBRK1.** *A*, schematic representation of ZBRK1 (the KRAB domain and numbered zinc fingers are indicated), MBP-ZBRK1 ΔK, and MBP-ZBRK1 ΔK truncation derivatives. *B*, purified MBP-ZBRK1 ΔK and its corresponding truncation derivatives as indicated were resolved by SDS-10% PAGE and visualized by Coomassie Blue staining. Molecular weight marker positions (M_r) are indicated. *C*, competition EMSA. EMSA was performed using a ^{32}P-labeled double-stranded oligonucleotide probe corresponding to a wild-type consensus ZRE and purified MBP-ZBRK1 ΔK (*1st lane*). A 100-, 200-, or 400-fold molar excess of an unlabeled wild-type (*WT*) ZRE probe (*2nd to 4th lanes*) or mutated (*Mut.*) ZRE probe corresponding to a double-stranded oligonucleotide identical in length but different in sequence (*5th to 7th lanes*) was added to the binding reaction as indicated. The positions of the unbound ZRE oligonucleotide probe (*ZRE*) and the ZRE·MBP-ZBRK1 ΔK nucleoprotein complex (*ZRE-ZBRK1*) are indicated. For each EMSA reaction, the proportional fraction of ZRE probe bound by MBP-ZBRK1 ΔK is indicated *below* each lane and was determined by dividing the number of radioactive counts in the bound probe by the number of radioactive counts in the bound plus the unbound probe. *D* and *E*, sequence-specific DNA binding activity of MBP-ZBRK1 ΔK and its corresponding truncation derivatives. EMSA was performed using the consensus ZRE probe and 50 ng of either MBP-ZBRK1 ΔK or each of its corresponding deletion derivatives as indicated. *NP* indicates no protein added to the EMSA reaction. The positions of the unbound ZRE oligonucleotide probe (*ZRE*) and the ZRE·MBP-ZBRK1 ΔK nucleoprotein complex (*ZRE-ZBRK1*) are indicated. DNA-binding reactions were performed in 100 mM NaCl (*D*) or 200 mM NaCl (*E*). For each EMSA reaction, the proportional fraction of ZRE probe bound by MBP-ZBRK1 ΔK or its truncation derivatives is indicated *below* each lane and was determined by dividing the number of radioactive counts in the bound probe by the number of radioactive counts in the bound plus the unbound probe. *ND*, not detectable.

$ZnCl_2$; and 10 mM β-mercaptoethanol) supplemented with protease inhibitors (aprotinin 0.4 μg/ml; chymostatin 0.5 μg/ml; leupeptin 0.5 μg/ml; pepstatin 0.5 μg/ml; PMSF 0.5 mM; and benzamidine-HCl 0.5 mM). Resuspended cells were frozen and thawed one time, followed by sonication (3 times for 1 min) and clarification by centrifugation at 30,000 × *g* for 30 min. MBP-ZBRK1 fusion proteins were purified from clarified lysates by affinity chromatography on amylose resin (New England Biolabs, Beverly, MA). Briefly, clarified lysates were incubated with amylose resin in batch for 1 h at 4 °C, washed 3 times with MBP binding buffer, and then eluted with MBP binding buffer containing 0.5% maltose for 30 min at 4 °C. Purified proteins (estimated to be >95% homogeneous by SDS-PAGE and subsequent visualization by Coomassie Blue staining) were dialyzed for 1 h at 4 °C against EMSA storage buffer (25 mM Tris-HCl, pH 7.5; 100 mM NaCl; 20 μM $ZnCl_2$; 10% glycerol; 10 mM β-mercaptoethanol; and 0.5 mM PMSF) before long term storage at −80 °C.

Electrophretic Mobility Shift Assay (EMSA)

For EMSA, the ZRE probe was obtained by annealing two complementary oligonucleotides corresponding in sequence to the consensus ZBRK1 DNA-binding site: 5′-GATCCACGGGACGCAGGTGTTTTGTGCCG-3′ and 5′-GATCCGGCACAAAACACCTGCGTCCCGTG-3′ (39). Mutant (Mut) probe was obtained by annealing two oligonucleotides, 5′-GATCCACCTCACGTTCGTGCACTGTGCCG-3′ and 5′-GATCCGGCACAGTGCACGAACGTGAGGTG-3′ (39). Each of these double-stranded probes carried overhanging ends, which were filled in with [α-^{32}P]dCTP by Klenow enzyme. In each reaction, purified MBP-ZBRK1 fusion proteins (50 ng) were incubated with 6000 cpm of a ^{32}P-labeled double-stranded oligonucleotide probe in 30 μl of EMSA binding buffer (25 mM Tris-HCl pH 7.5; 20 μM $ZnCl_2$; 12.5% glycerol; 0.5 mM PMSF; and a variable concentration of NaCl as indicated). Following 30 min of incubation at room temperature, reaction mixtures were loaded onto a 5% non-denaturing polyacrylamide gel and electrophoresed at 200 V for 2 h at 4 °C in 0.5× TBE. Dried gels were subjected to PhosphorImager analysis (Amersham Biosciences).

Cell Culture, Transfections, and Reporter Assays

Brca1−/−, p53−/− (*Brca1−/−*), and p53−/− (*Brca1+/+*) mouse embryo fibroblasts (MEFs) (39) and U2OS human osteosarcoma cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). *Brca1+/+*, *Brca1−/−*, and U2OS cells were transfected at 60% confluency using Effectene reagent (Qiagen, Valencia, CA), and the expression and reporter plasmids are indicated in each figure. Each transfection also included an internal control plasmid, pCH110 (40), expressing β-galactosidase under control of the SV40 promoter. Forty-eight hours

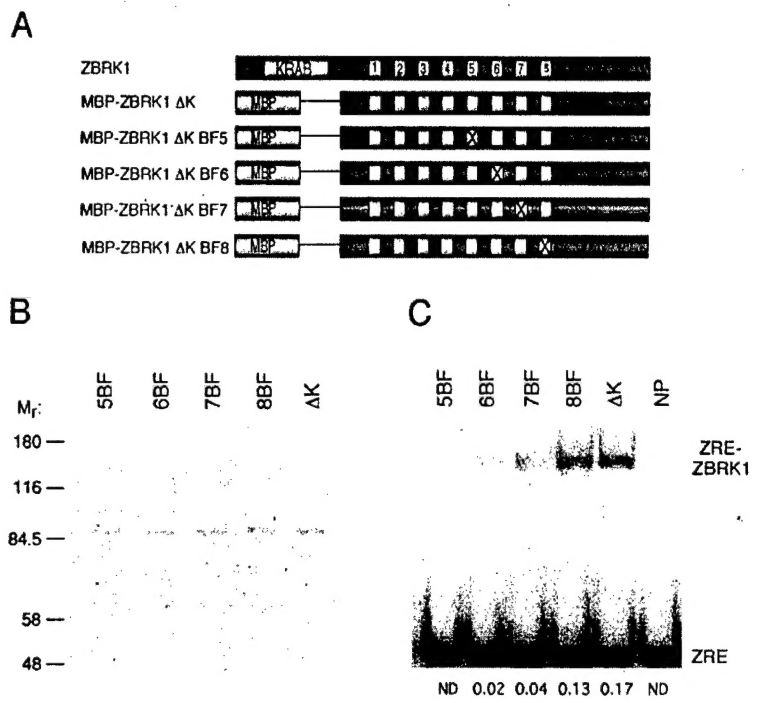

FIG. 2. **Identification of DNA-binding determinants on ZBRK1.** *A*, schematic representation of ZBRK1, MBP-ZBRK1 ΔK, and MBP-ZBRK1 ΔK broken finger derivatives. *B*, purified MBP-ZBRK1 ΔK and its corresponding broken finger derivatives as indicated were resolved by SDS-10% PAGE and visualized by Coomassie Blue staining. Molecular weight marker positions (M_r) are indicated. *C*, sequence-specific DNA binding activity of MBP-ZBRK1 ΔK and its corresponding broken finger derivatives. EMSA was performed using the consensus ZRE probe and 50 ng of either MBP-ZBRK1 ΔK or each of its corresponding broken finger derivatives as indicated. *NP* indicates no protein added to the EMSA reaction. The positions of the unbound ZRE oligonucleotide probe (*ZRE*) and the ZRE·MBP-ZBRK1 ΔK nucleoprotein complex (*ZRE-ZBRK1*) are indicated. DNA-binding reactions were performed in 200 mM NaCl. For each EMSA reaction, the proportional fraction of ZRE probe bound by MBP-ZBRK1 ΔK or its broken finger derivatives is indicated *below* each lane and was determined by dividing the number of radioactive counts in the bound probe by the number of radioactive counts in the bound plus the unbound probe. *ND*, not detectable.

post-transfection, cells were harvested and lysed in Reporter Lysis buffer (Promega, Madison, WI). Transfected cell lysates (20 μl) were analyzed for luciferase activity using the luciferase assay system (Promega, Madison, WI) and for β-galactosidase activity using the Galacto-light Plus Chemiluminescent Reporter Assay (BD Biosciences). Each transfection was repeated a minimum of 3 times in duplicate.

Yeast Two-hybrid Interaction Assay

pGADT7-ZBRK1 5ZFC and its truncation (6ZFC, 7ZFC, 8ZFC, 0ZFC, 5ZFC Δ1, 5ZFC Δ2, and 5ZFC Δ3) and broken finger (BF5, BF6, BF7, and BF8) derivatives were individually co-transformed along with pGBKT7-BRCA1 (expressing BRCA1 amino acids 341–748) into the yeast strain Y187 (BD Biosciences/Clontech). After selection, colonies were expanded in liquid culture for assay of β-galactosidase activity following previously established procedures (39).

Transient Expression Analysis

Steady-state levels of GAL4-ZBRK1 5ZFC protein and its truncation and substitution derivatives were comparatively analyzed by immunoblot analysis of transfected whole cell extracts in order to verify equivalent levels of ectopic protein expression. Briefly, U2OS cells transfected with GAL4-ZBRK1 5ZFC, truncation mutants GAL4-ZBRK1 6ZF, 7ZFC, 8ZFC, 0ZFC, 5ZFC Δ1, 5ZFC Δ2, and 5ZFC Δ3, and broken finger mutants GAL4-ZBRK1 5ZFC BF5, BF6, BF7, and BF8 were lysed in Laemmli sample buffer, resolved by SDS-10% PAGE, and subjected to immunoblot analysis using an antibody directed against the GAL4 DNA-binding domain (sc-510, Santa Cruz Biotechnology, Santa Cruz, CA) and, as an internal control protein, the p89 subunit of TFIIH (sc-293, Santa Cruz Biotechnology, Santa Cruz, CA). Immunodetection was performed using ECL Western blotting detection reagents (Amersham Biosciences).

RESULTS

Identification of DNA-binding Determinants on ZBRK1—Previously, we demonstrated a strict requirement for BRCA1 in ZBRK1 repression. Specifically, we showed that ZBRK1 repression function was similarly abrogated by genetic ablation of *Brca1* or by deletion of the BRCA1-binding domain on ZBRK1 (39). The BRCA1-binding domain on ZBRK1 includes the last four of eight ZBRK1 zinc fingers (zinc fingers 5–8) along with the ZBRK1 C terminus (39). Whether and how this zinc finger domain contributes to the sequence-specific DNA binding activity of ZBRK1, however, is presently unknown. Because the specification of individual zinc fingers required to bind to DNA and/or BRCA1 could illuminate the underlying mechanism(s) by which BRCA1 mediates ZBRK1 repression, we initially sought to establish both the number and identity of the ZBRK1 zinc fingers required to bind DNA and BRCA1.

With respect to DNA binding, we showed previously (39) that the eight central ZBRK1 zinc fingers collectively recognize a 15-bp consensus sequence, GGG*xxx*CAG*xxx*TTT. Based on the observation that one C_2H_2 zinc finger can bind to ~3 bp of DNA (52–54), only five of the eight ZBRK1 zinc fingers would be predicted to bind to its 15-bp consensus sequence. To test this prediction, we analyzed a panel of ZBRK1 zinc finger deletion derivatives for their respective abilities to bind to the consensus ZBRK1 DNA-binding site in an EMSA.

To this end, we expressed ZBRK1 as a maltose-binding protein (MBP) chimera in *E. coli*, which permitted the purification of otherwise insoluble ZBRK1 protein. Full-length MBP-ZBRK1 is expressed poorly, whereas MBP-ZBRK1 ΔK (a deletion derivative lacking the N-terminal 143 amino acids of the 532-amino acid full-length ZBRK1 protein) is abundantly expressed. ZBRK1 ΔK lacks the N-terminal KRAB domain but retains the ZBRK1 zinc fingers and the C terminus, elements that are required for binding to both DNA and/or BRCA1 (Fig. 1, *A* and *B*). We have therefore utilized this recombinant ZBRK1 derivative as the background into which truncation and substitution mutations have been introduced for purposes of DNA binding assays.

In an EMSA, MBP-ZBRK1 ΔK produced a discrete nucleoprotein complex on a double-stranded oligonucleotide probe corresponding to the consensus ZBRK1-response element (ZRE); a molar excess of unlabeled WT ZRE probe (WT probe), but not a mutant probe (Mut probe), efficiently competed for the formation of this complex, thus establishing sequence-specific DNA binding by MBP-ZBRK1 ΔK in this assay (Fig. 1*C*). To determine the number and identity of the ZBRK1 zinc fingers required to bind to its consensus sequence, we analyzed a series of C-terminal truncation derivatives bearing stepwise deletions of individual ZBRK1 zinc fingers (Fig. 1, *A* and *B*). In

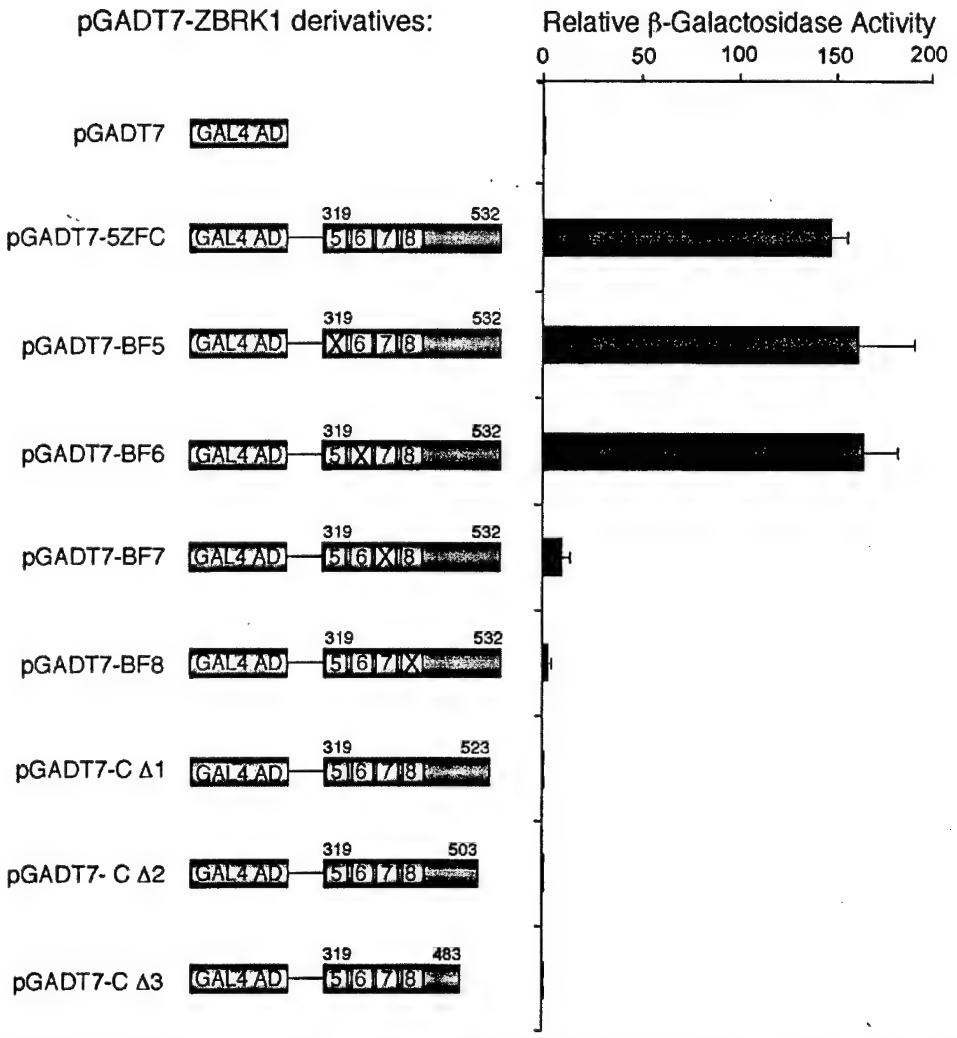

FIG. 3. **Identification of BRCA1-binding determinants on ZBRK1.** The ZBRK1-binding region on BRCA1 (amino acids 341–748) (39), expressed as a GAL4 DNA-binding domain fusion protein (in the plasmid pGBKT7), was tested for interaction with the indicated fragments of ZBRK1 fused to the GAL4 transactivation domain (plasmid pGADT7) in yeast two-hybrid assays. β-Galactosidase activities were quantified as described previously (39). Corresponding β-galactosidase activities obtained with each pGADT7-ZBRK1 5ZFC derivative are expressed relative to that observed with the backbone pGADT7 expression vector alone, which was arbitrarily assigned a value of 1. Values represents the average of three independent assays, each performed in triplicate, and *error bars* represent the mean ± S.D. As a comparative measure of interaction strength, the average β-galactosidase activity obtained with pGBKT7-p53 and pGADT7-Large T antigen was 512 ± 13.5. The validity of two-hybrid interactions observed in these experiments was further substantiated by the following controls. First, we confirmed that the observed β-galactosidase activities were dependent upon BRCA1 sequences expressed from the plasmid pGBKT7-BRCA1 by performing a parallel series of interaction assays with individual pGADT7-ZBRK1 5ZFC derivatives and the backbone vector pGBKT7 as a negative control. Second, immunoblot analyses of yeast whole cell extracts confirmed that each of the pGADT7-ZBRK1 5ZFC fusion proteins was expressed at roughly equivalent levels, thus excluding the possibility that differences in β-galactosidase activities derive from difference in fusion protein expression.

100 mM NaCl, truncation of the ZBRK1 C terminus (MBP-ZBRK1 ΔK 8ZF) did not appreciably affect DNA binding relative to intact MBP-ZBRK1 ΔK (Fig. 1*D*). Interestingly, deletion of the eighth and last ZBRK1 zinc finger along with the C terminus (MBP-ZBRK1 ΔK 7ZF) led to an increase in DNA binding activity, suggesting that ZBRK1 ZF8, and possibly the C terminus, constrains sequence-specific DNA binding mediated by the first seven zinc fingers (Fig. 1*D*). This effect was exacerbated under more stringent DNA binding conditions (200 mM NaCl) (Fig. 1*E*). At 100 mM NaCl, stepwise truncation of zinc fingers 7 to 5 led to a slight incremental reduction in sequence-specific DNA binding activity (Fig. 1*D*). ZBRK1 derivatives bearing less than four zinc fingers failed to bind to DNA, thereby establishing zinc fingers 1–4 as the minimal ZBRK1 DNA-binding domain under these conditions (Fig. 1*D*). Identical results were observed at 50 mM NaCl (data not shown). At 200 mM NaCl, zinc fingers 1–5 were required for stable DNA binding, and the inclusion of zinc fingers 6 and 7 incrementally stabilized binding (Fig. 1*E*).

To examine more rigorously the role of zinc fingers 5–8 in sequence-specific DNA binding by ZBRK1, we examined a set of "broken finger" mutants bearing substitution mutations within each of these zinc fingers. This approach permitted us to assess the individual contribution of each finger within the BRCA1-binding domain to overall DNA binding activity in the context of the eight-fingered ZBRK1 ΔK protein and thereby circumvent potential artifacts arising from analyses of truncation mutants. Each broken finger mutant bears a His-to-Asn substitution mutation at the first of the two conserved His residues within the targeted C_2H_2 zinc finger (Fig. 2, *A* and *B*). The relative conservative nature of this substitution eliminates zinc coordination within the targeted finger, thereby disrupting

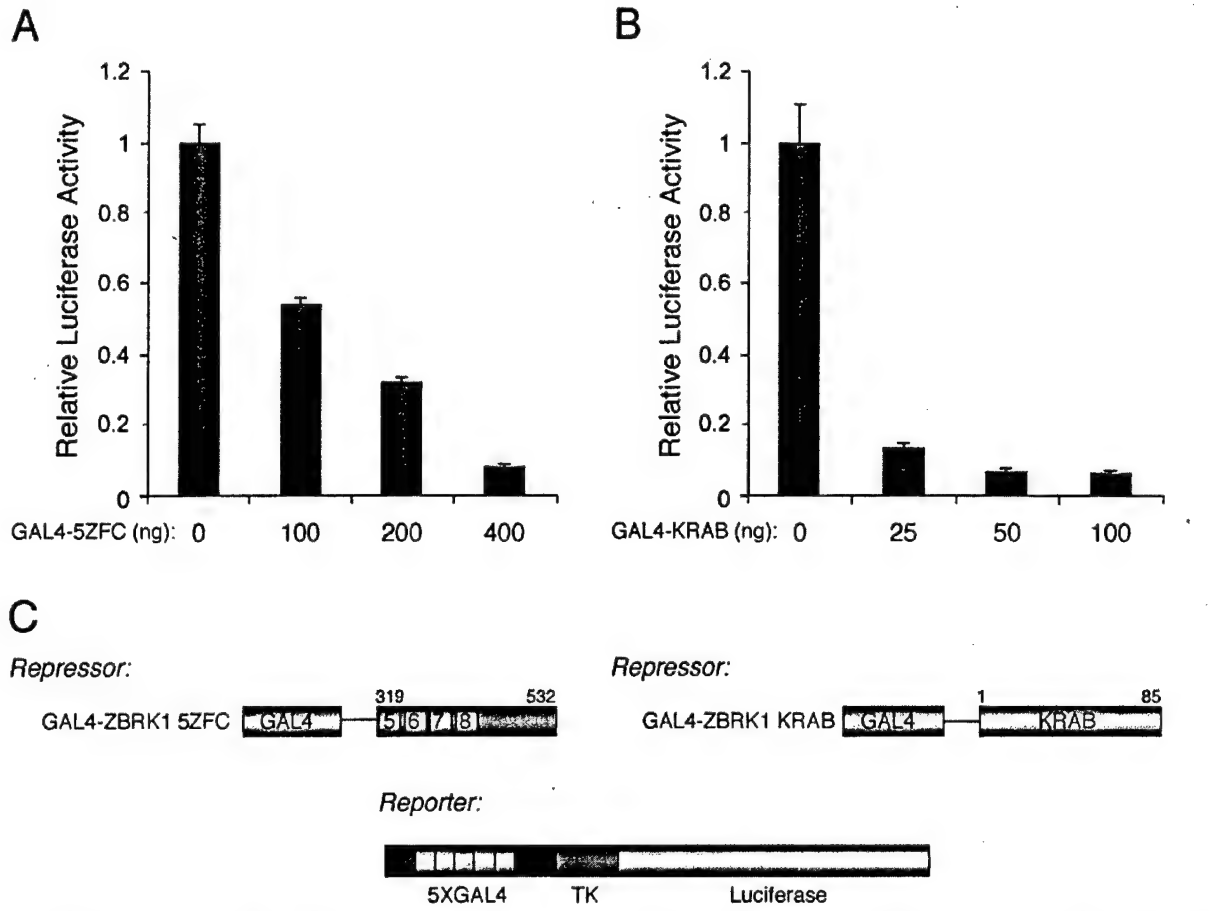

FIG. 4. **ZBRK1 harbors two independent transcriptional repression domains.** *A* and *B*, human U2OS cells were transfected with 30 ng of pG_5TK-Luc bearing five copies of the GAL4 DNA-binding site sequence upstream of the TK promoter without or with the indicated nanogram amounts of GAL4-ZBRK1 5ZFC (*A*) or GAL4-KRAB (*B*). In this and all subsequent transfection experiments involving effector plasmid titrations, the total amount of DNA in each transfection was fixed by reciprocal titration of the corresponding backbone expression plasmid. Also, in this and all subsequent transfection experiments, the relative luciferase activity represents the ratio of the luciferase activity obtained in a particular transfection to that obtained in cells transfected with only the reporter and pM (GAL4 DNA-binding domain) expression vectors alone. Luciferase activities were first normalized to β-galactosidase activity obtained by co-transfection of the SV40-β-gal vector (15 ng) as described previously (39). *Error bars* represent the S.D. from the average of at least three independent transfections performed in duplicate. *C*, schematic representation of GAL4-ZBRK1 5ZFC and GAL4-ZBRK1 KRAB chimeras (amino acid sequences fused to the GAL4 DNA-binding domain are indicated *numerically above* each chimera) and the pG_5TK-Luc reporter template used in transfection assays.

its local structure with little effect on the integrity of the remainder of the protein (55, 56). Consistent with the results obtained using C-terminal truncation mutants, analysis of individual BF mutants 5–8 revealed zinc finger 5 to be an important ZBRK1 determinant for stable DNA binding, whereas zinc fingers 6 and 7 promote but are not essential for binding (Fig. 2*C*). Disruption of zinc finger 8 did not appreciably affect the DNA binding activity of MBP-ZBRK1 ΔK, suggesting that zinc finger 8 is largely dispensable for stable association with DNA (Fig. 2*C*). Furthermore, local disruption of zinc finger 8 did not relieve constraints on the DNA binding activity of MBP-ZBRK1 ΔK, suggesting that the C terminus of ZBRK1 can also mask the inherent DNA binding activity of ZBRK1 zinc fingers 1–7 (Fig. 2*C*). In summary, the results of DNA-binding analyses delimit the core ZBRK1 DNA-binding domain to zinc fingers 1–4; these zinc fingers are minimally required for stable DNA binding under relatively non-stringent conditions of ionic strength. Zinc finger 5 is a critical and context-dependent determinant of stable binding and represents the extent of the minimal DNA-binding domain under more stringent binding conditions. Zinc fingers 6 and 7, although nonessential, nonetheless further stabilize DNA binding mediated by zinc fingers 1–5. Finally, zinc finger 8 and the C terminus apparently destabilize the maximum potential DNA binding activity inherent in zinc fingers 1–7.

Identification of BRCA1-binding Determinants on ZBRK1—Next, we sought to establish more precisely the molecular determinants on ZBRK1 required for BRCA1 binding. Previously, we mapped the BRCA1-binding domain on ZBRK1 to encompass a broad region extending from zinc finger 5 through the C terminus (5ZFC) (39). To more narrowly define the BRCA1-binding determinants on ZBRK1, we examined the contribution of individual zinc fingers 5–8 as well as sequences within the ZBRK1 C terminus to BRCA1 binding using a yeast two-hybrid interaction assay. To this end, individual substitution and truncation mutations within ZBRK1 5ZFC were translationally fused to the GAL4 transactivation domain and tested for their respective abilities to bind to the ZBRK1-interaction domain on BRCA1 (amino acids 341–748), translationally fused to the GAL4 DNA-binding domain in yeast (Fig. 3). Corresponding β-galactosidase activities identified critical determinants of BRCA1 interaction on ZBRK1 to include the C terminus as well as zinc fingers 7 and 8 (Fig. 3). Deletion of only 9 amino acids from the C terminus significantly compromised BRCA1 binding, indicating that the unique C terminus on ZBRK1 is required in its entirety for efficient interaction with BRCA1 (Fig. 3). This observation sug-

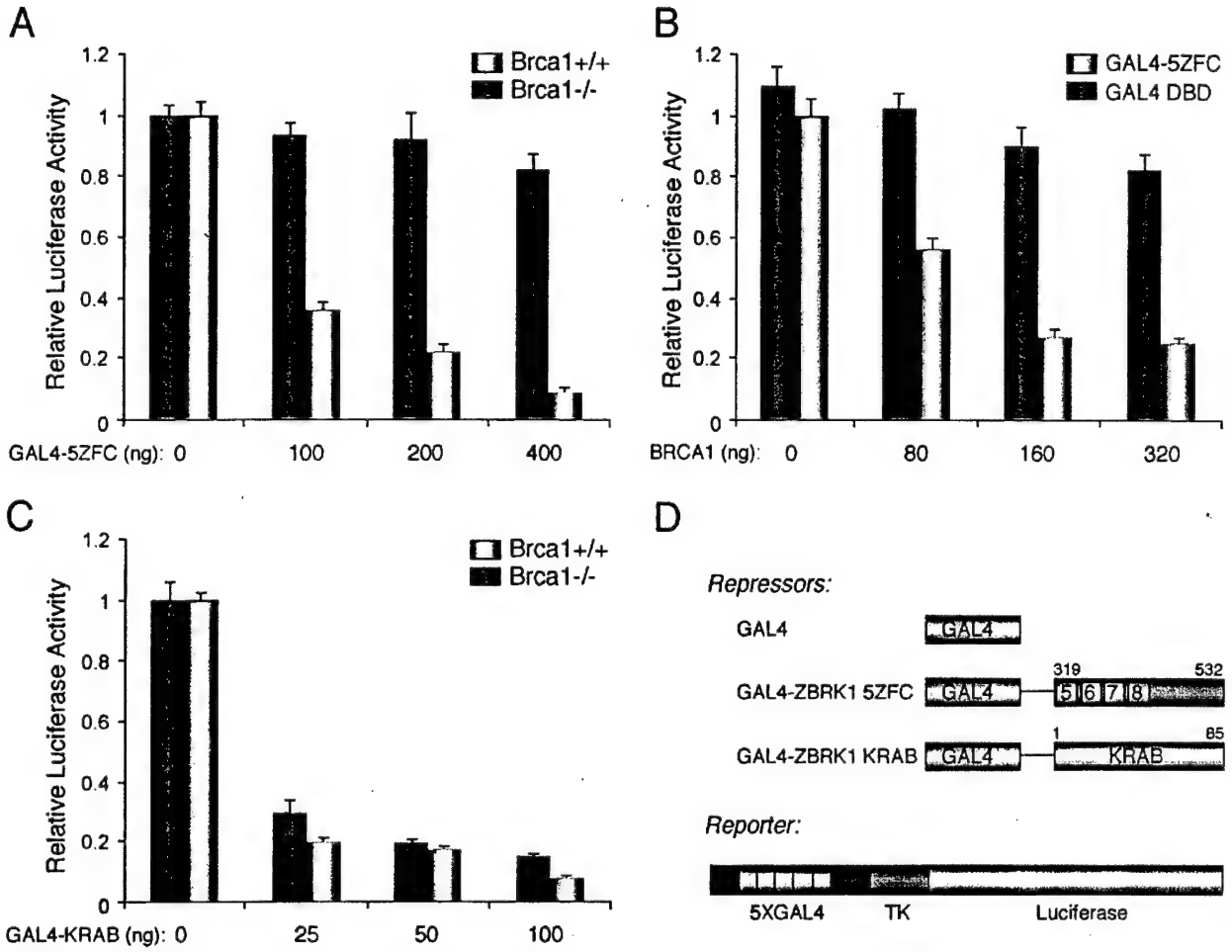

FIG. 5. **The ZBRK1 5ZFC and KRAB repression domains function in a BRCA1-dependent and BRCA1-independent manner, respectively.** *A*, *Brca1+/+* and *Brca1−/−* MEF cells (39) as indicated were transfected with 100 ng of pG_5TK-LUC without or with the indicated nanogram amounts of GAL4-ZBRK1 5ZFC. *B*, *Brca1−/−* MEF cells were transfected with 100 ng of pG_5TK-Luc and 20 ng of either pM (expressing the GAL4 DNA-binding domain (*DBD*) alone) or GAL4-ZBRK1 5ZFC, respectively, without or with the indicated nanogram amounts of pCS2+-BRCA1 expressing wild-type human BRCA1. *C*, *Brca1+/+* and *Brca1−/−* MEF cells as indicated were transfected with 100 ng of pG_5TK-Luc without or with the indicated nanogram amounts of GAL4-ZBRK1 KRAB. *A–C*, relative luciferase activities were calculated as described in the legend to Fig. 4. *D*, schematic representation of the GAL4 DNA-binding domain, the GAL4-ZBRK1 5ZFC and GAL4-ZBRK1 KRAB chimeras, and the pG_5TK-Luc reporter template used in transfection assays.

gests that the overall conformation of the C terminus is likely to be important for BRCA1 interaction. Whereas ZBRK1 zinc fingers 7 and 8 are critical for BRCA1 interaction, zinc fingers 5 and 6 do not appear to contribute to BRCA1 binding (Fig. 3). Taken together, these results indicate that important BRCA1-binding determinants on ZBRK1 include those that also modulate its sequence-specific DNA binding activity in both a positive (zinc finger 7) and negative (zinc finger 8 and the C terminus) manner.

The BRCA1-binding Domain on ZBRK1 Functions as an Autonomous BRCA1-dependent Transcriptional Repression Domain—The fact that BRCA1 contacts ZBRK1 through surfaces that are not essential but nonetheless modulatory with respect to DNA binding suggests several potential mechanisms by which BRCA1 might mediate transcriptional repression by ZBRK1. First, BRCA1 could mediate ZBRK1 repression, at least in part, by modulating its sequence-specific association with DNA. This possibility is currently under investigation. Alternatively, or additionally, BRCA1 could mediate repression by DNA-bound ZBRK1. This possibility is supported by our previous observation that clinically validated missense mutations within the BRCA1 C terminus that do not disrupt its interaction with ZBRK1 nonetheless abrogate its ZBRK1 co-repressor activity (39). To test this possibility directly, we examined whether the BRCA1-binding domain on ZBRK1 could function as a BRCA1-dependent transcriptional repression domain when tethered to a heterologous DNA-binding domain. This approach permitted us to assess the influence of BRCA1 on the repression function of ZBRK1 independently of any effects that it might have on the DNA binding activity of ZBRK1. Accordingly, we initially tested the ability of ZBRK1 5ZFC (extending from zinc finger 5 to the C terminus) to function as an independent repression domain when linked to the GAL4 DNA-binding domain. GAL4-ZBRK1 5ZFC was transiently expressed in U2OS human osteosarcoma cells, and its influence on transcription from a pG_5TK-Luc reporter template bearing five copies of the consensus GAL4 DNA-binding site upstream of the herpes simplex virus (HSV) TK gene promoter was examined. GAL4-ZBRK1 5ZFC conferred greater than 10-fold repression upon reporter gene expression in a dose-dependent manner (Fig. 4*A*). We also confirmed the presence of a potent KRAB repression domain within the ZBRK1 N terminus by examining its ability repress pG_5TK-Luc reporter gene expression when tethered to the GAL4 DNA-binding domain (Fig. 4*B*). Based on quantitative immunoblot analysis of transfected

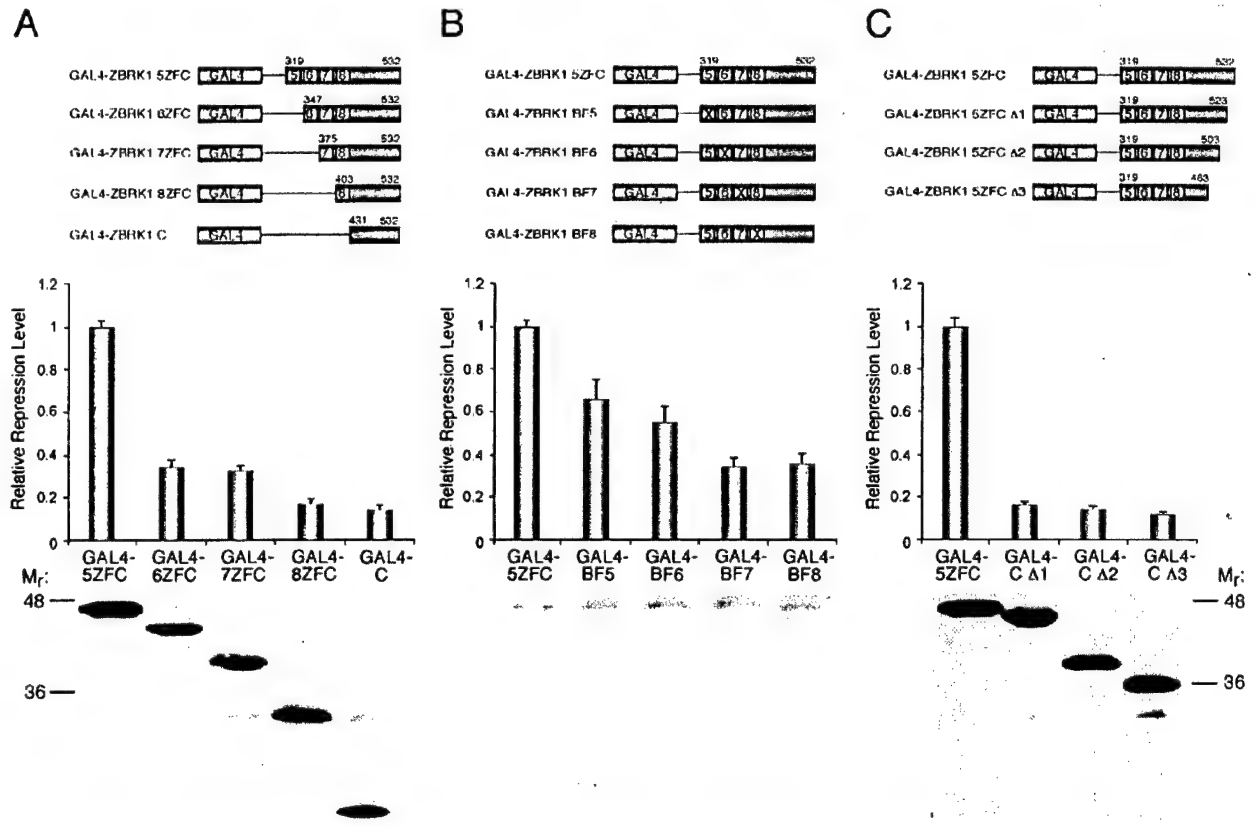

FIG. 6. **Functional analysis of ZBRK1 5ZFC truncation and substitution derivatives.** *A–C*, U2OS cells were transfected with 30 ng of pG_5TK-Luc and the following amounts of GAL4-ZBRK1 5ZFC or its indicated derivatives: *A*, 5ZFC, 400 ng; 6ZFC, 7ZFC, 8ZFC, and C, 200 ng; *B*, 5ZFC or its indicated broken finger derivatives, 200 ng; *C*, 5ZFC, 400 ng; Δ1, 200 ng; Δ2, 400 ng; Δ3, 200 ng. In each panel, the relative repression level represents the relative luciferase activity obtained with a particular GAL4-ZBRK1 5ZFC derivative divided by that obtained with GAL4-ZBRK1 5ZFC. Relative luciferase activities were calculated as described in the legend to Fig. 4. In these experiments, the relative luciferase activity observed with GAL4-ZBRK1 5ZFC (three independent transfections performed in duplicate) was as follows: *A*, 0.10 corresponding to 10-fold repression of reporter template activity; *B*, 0.21 corresponding to an approximate 5-fold repression of reporter template activity; *C*, 0.08 corresponding to 12.5-fold repression of reporter template activity. To confirm that GAL4-ZBRK1 5ZFC and its truncation and substitution derivatives are expressed at roughly equivalent levels, lysates from U2OS cells transfected with GAL4-ZBRK1 5ZFC or its derivatives (the same amounts used in functional analysis and indicated above) were resolved by SDS-10% PAGE and subjected to immunoblot analysis using antibodies specific for the GAL4 DNA-binding domain. Molecular weight marker positions (M_r) are indicated. GAL4-ZBRK1 5ZFC and its derivatives are represented schematically at the *top* of each panel.

cell extracts, the ZBRK1 KRAB domain, on a molar basis, appears to be a stronger transcriptional repression domain than the ZBRK1 BRCA1-binding domain (data not shown). Nonetheless, this result indicates the presence within ZBRK1 of two portable transcriptional repression domains, an N-terminal KRAB domain and a novel C-terminal transcriptional repression domain (5ZFC) that encompasses the BRCA1-binding domain.

To confirm the requirement for BRCA1 in transcriptional repression by ZBRK1 5ZFC, we tested the repression function of GAL4-ZBRK1 5ZFC in *Brca1+/+* and *Brca1−/−* MEF cells. GAL4-ZBRK1 5ZFC conferred up to 10-fold repression in *Brca1+/+* MEFs, whereas little or no repression activity was observed in *Brca1−/−* MEFs (Fig. 5*A*). Ectopic expression of BRCA1 in *Brca1−/−* MEFs restored ZBRK1 5ZFC-directed transcriptional repression (Fig. 5*B*), establishing conclusively that BRCA1 mediates repression by DNA-bound ZBRK1 5ZFC. In contrast to ZBRK1 5ZFC, the ZBRK1 KRAB domain repressed transcription equivalently in both *Brca1+/+* and *Brca1−/−* MEFs (Fig. 5*C*). Thus, the N-terminal KRAB and C-terminal 5ZFC repression domains within ZBRK1 can be distinguished functionally on the basis of their respective requirements for BRCA1.

BRCA1-binding Is Necessary but Not Sufficient for ZBRK1 5ZFC Repression Function—To more narrowly define the boundaries of the BRCA1-dependent 5ZFC repression domain within ZBRK1, we examined a panel of ZBRK1 5ZFC truncation and substitution mutants for their respective repression activities *in vivo*. Relative to the intact 5ZFC domain, deletion or disruption of zinc fingers 5 or 6 individually reduced repression activity by 2–3-fold (Fig. 6, *A* and *B*), whereas individual deletion or disruption of zinc fingers 7 or 8 reduced repression activity by 3–6-fold (Fig. 6, *A* and *B*). These results indicate that zinc fingers 5–8 are all required for the integrity of the 5ZFC repression domain, although zinc fingers 7 and 8 appear to be quantitatively more important. Deletion of only 9 amino acids from the C terminus of ZBRK1 severely compromised the repression function of the 5ZFC domain, indicating that the entire C terminus is likely to be important for 5ZFC repression activity (Fig. 6*C*). Thus, the 5ZFC repression domain extending from ZBRK1 zinc finger 5 through the C terminus appears to constitute an intact repression domain that cannot be further delimited. This analysis also reveals an imperfect correlation between BRCA1 binding and transcriptional repression by the 5ZFC repression domain. Thus, disruption of ZBRK1 zinc fingers 7 or 8 or truncation of the C terminus severely compromised BRCA1 binding (Fig. 3) and transcriptional repression (Fig. 6). By contrast, disruption of zinc fingers 5 or 6, which are

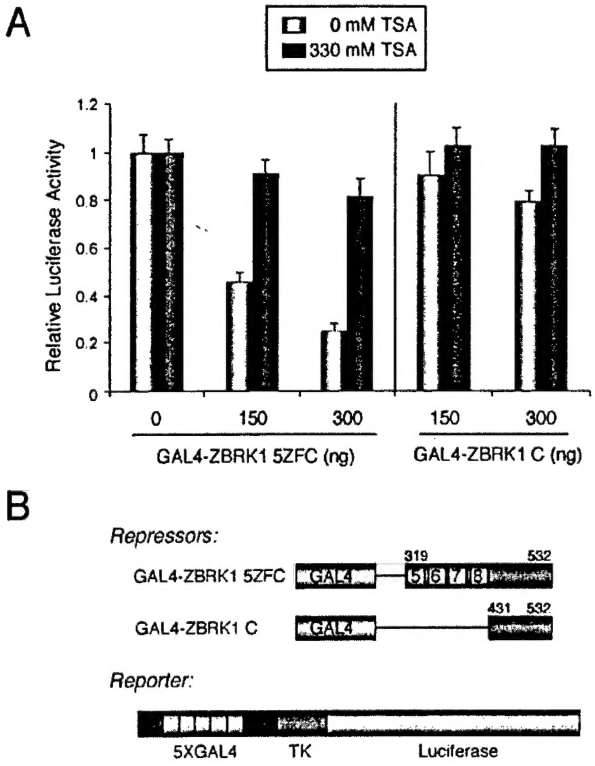

FIG. 7. **ZBRK1 5ZFC-directed repression is reversed by trichostatin A.** *A*, U2OS cells were transfected with 30 ng of pG_5TK-Luc without or with the indicated nanogram amounts of GAL4-ZBRK1 5ZFC or GAL4-ZBRK1 C. Where indicated, TSA (330 nM) was also included. Relative luciferase activities were calculated as described in the legend to Fig. 4. *B*, schematic representation of the GAL4-ZBRK1 5ZFC and GAL4-ZBRK1 C chimeras and the pG_5TK-Luc reporter template used in transfection assays.

not required for BRCA1 binding (Fig. 3), nonetheless significantly compromised transcriptional repression (Fig. 6, *A* and *B*). On this basis we conclude that BRCA1 binding is necessary but not sufficient for 5ZFC repression function. This suggests that ZBRK1 zinc fingers 5 and 6 may possibly contact an additional co-repressor(s).

The BRCA1-dependent 5ZFC Repression Domain Is Histone Deacetylase-dependent and Promoter-specific—Previously, BRCA1 has been shown to interact through its C-terminal BRCT repeats with histone deacetylases (HDACs) 1 and 2 (57). HDACs remove acetyl groups from lysine residues on histone tails and thus promote the formation of transcriptionally repressive chromatin. To determine the contribution of HDAC activity to repression mediated by ZBRK1 5ZFC, we tested the effect of the selective HDAC inhibitor trichostatin A (TSA) on repression mediated by GAL4-ZBRK1 5ZFC. TSA largely reversed GAL4-ZBRK1 5ZFC repression in U2OS cells, implicating HDAC activity in this process (Fig. 7).

Like the ZBRK1 5ZFC repression domain, KRAB repression domains function through HDACs as well as through histone methyltransferases and heterochromatin proteins (41–47). This prompted us to comparatively examine the promoter specificities of the ZBRK1 5ZFC and KRAB repression domains. The ZBRK1 N-terminal KRAB domain repressed transcription potently from each of three different RNA polymerase II promoters tested: the SV40 major late, the HSV TK, and the human small nuclear ribonucleoprotein N (SNRPN) promoters (Fig. 8*B*). By contrast, ZBRK1 5ZF5C repressed transcription potently from the HSV TK promoter, moderately from the SV40 promoter, and not at all from the SNRPN promoter (Fig. 8A). These results indicate that the ZBRK1 KRAB and 5ZFC repression domains can be distinguished functionally not only by their requirement for BRCA1 but also on the basis of their promoter specificities; whereas the ZBRK1 KRAB repression domain exhibits broad promoter selectivity, the BRCA1-dependent 5ZFC repression domain exhibits a more restricted promoter bias.

DISCUSSION

A central question regarding the role of BRCA1 in transcription control concerns the means by which it mediates gene-specific regulation in the absence of sequence-specific DNA binding activity. In part, this question has been answered by the identification of a growing number of sequence-specific DNA-binding transcription factors with which BRCA1 physically and functionally interacts. In this regard, our previous identification of ZBRK1 as a BRCA1-dependent transcriptional repressor provided a molecular basis to link BRCA1 directly to the regulation of *GADD45a* gene expression (39). Other work has rendered it clear that the BRCA1 regulation of *GADD45a* gene transcription is likely to be complex and mediated not only through ZBRK1 but other trans-acting factors, including OCT1 and NF-YA (21, 32, 39). Presently, however, little is known regarding the mechanism(s) by which BRCA1 mediates sequence-specific transcriptional control through the various transcription factors with which it interacts. Here we have investigated the functional interaction between ZBRK1 and BRCA1 in an effort to understand the role of BRCA1 in sequence-specific transcriptional repression.

Our studies suggest that BRCA1 mediates ZBRK1 repression, at least in part, through its targeted recruitment to a novel C-terminal repression domain (5ZFC) within ZBRK1. Structurally, this repression domain comprises the last four zinc fingers and the unique C-terminal extension of ZBRK1. The identification of 5ZFC as a discrete functional domain was revealed by its ability to repress transcription when tethered to a heterologous DNA-binding domain (Fig. 4) and its functional resistance to truncation or substitution mutagenesis (Fig. 6). Importantly, we demonstrated that 5ZFC repression function is dependent upon BRCA1; genetic ablation of *Brca1* or disruption of BRCA1-binding determinants on 5ZFC similarly abrogates the repression function of this domain (Figs. 5 and 6). The functional contribution of this domain to BRCA1-dependent ZBRK1 repression is reflected by our previous observation that deletion of the ZBRK1 C terminus abrogates ZBRK1 repression through natural ZBRK1-response elements (39). However, whereas BRCA1 binding is necessary, our studies here suggest that it is not sufficient for 5ZFC-directed repression. First, BRCA1 binding and repression determinants within this domain can be separated, suggesting a possible functional requirement for a co-repressor(s) in addition to BRCA1 (Figs. 3 and 6). Second, 5ZFC-directed repression is HDAC-dependent (Fig. 7). Thus, we propose that the ZBRK1 5ZFC repression domain recruits BRCA1 as part of a higher order repression complex that minimally includes an associated HDAC activity. Targeted attempts to identify the functionally relevant BRCA1-associated co-repressor activities are currently underway.

Our work further reveals unique insight into the structural and functional organization of ZBRK1, a member of the KRAB-ZFP family. The ~220 members of this family make up a significant proportion of the transcription factor complement of the human proteome and are believed to occupy important regulatory roles in development, differentiation, and transformation (41, 58–63). Despite their potential biological significance, our current understanding of the mechanisms through

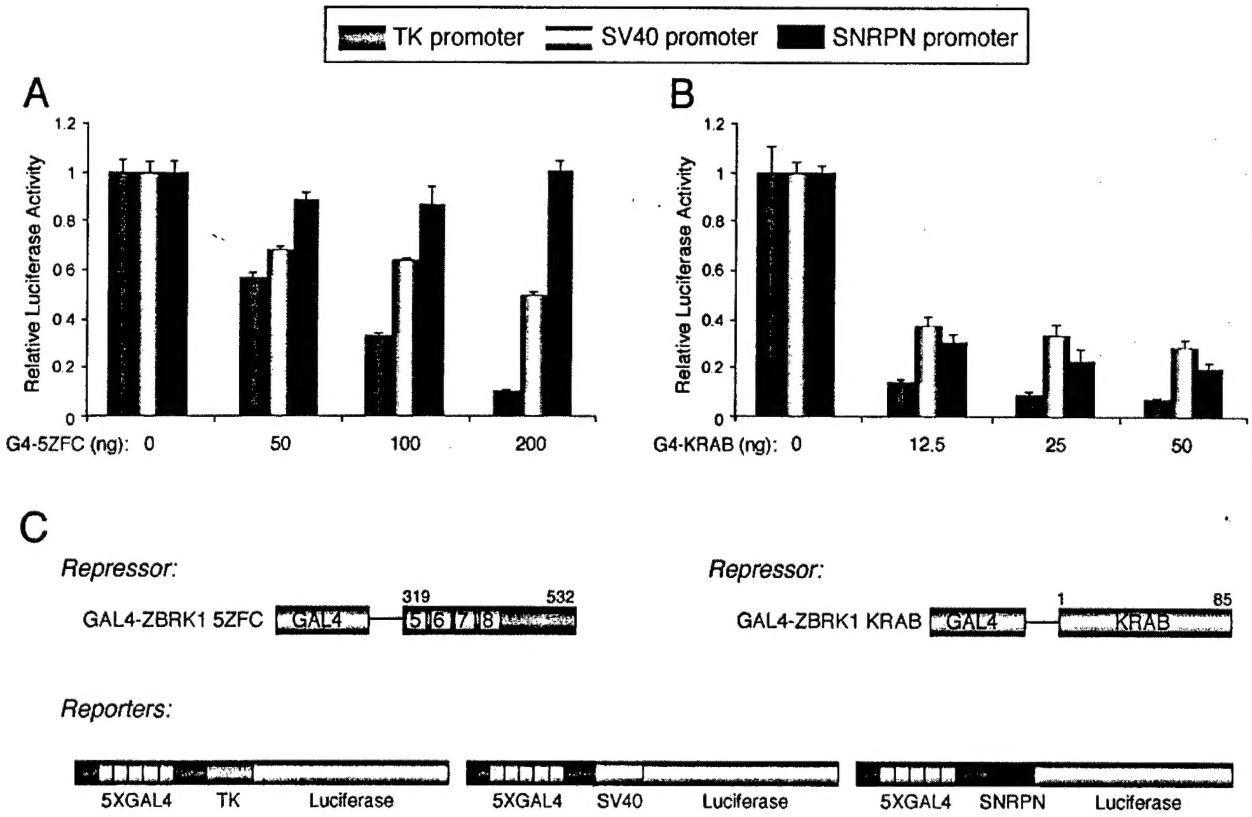

FIG. 8. **The ZBRK1 KRAB and 5ZFC repression domains exhibit unique promoter specificities.** *A* and *B*, U2OS cells were transfected with 30 ng of pG_5TK-Luc, 15 ng pG_5SV40-Luc, or 90 ng pG_5SNRPN-Luc as indicated without or with the indicated nanogram amounts of GAL4-ZBRK1 5ZFC (*A*) or GAL4-ZBRK1 KRAB (*B*). Relative luciferase activities were calculated as described in the legend to Fig. 4. *C*, schematic representation of the GAL4-ZBRK1 KRAB and GAL4-ZBRK1 5ZFC chimeras, and the pG_5TK-Luc, pG_5SV40-Luc, and pG_5SNRPN-Luc reporter templates used in transfections.

which individual members of this protein family function is still rather limited. Thus, although considerable mechanistic insight into the repression function of the KRAB domain has been revealed in recent years (41–47), comparatively little is known regarding the role of KRAB domain-associated zinc fingers in transcriptional repression apart from their presumed role in sequence-specific DNA binding. In part, this gap in knowledge derives from the limited availability of KRAB-ZFP target sequences with which structure-function analyses may be carried out. In the case of several KRAB-ZFPs whose corresponding binding site sequences have been identified, an additional function(s) for individual zinc fingers beyond DNA binding seems implicit. For example, based on the observation that one C_2H_2 zinc finger can bind to ~3 bp of DNA (52–54), the established target sequence lengths of 5 and 27 bp, respectively, for the 8- and 10-fingered ZNF202 and KS1 proteins are incompatible with DNA contact mediated by every zinc finger (61, 63). Our previous derivation of a consensus binding sequence for ZBRK1 has permitted us here to dissect a long array KRAB-ZFP and examine the contribution of individual zinc fingers to both sequence-specific DNA-binding and transcriptional repression. Our studies reveal the ZBRK1 zinc fingers to be multifunctional in nature, with dedicated roles in binding DNA, BRCA1, or both.

First, zinc fingers 1–4 are essential for DNA binding activity and compose the minimal DNA-binding domain under moderate conditions of ionic strength (Fig. 1). Zinc finger 5 appears to be a critical and context-dependent DNA-binding zinc finger; this finger represents the extent of the minimal DNA-binding domain under more stringent conditions (Fig. 1). Zinc fingers 6 and 7 are not essential for DNA binding but nonetheless enhance the stability of DNA binding (Figs. 1 and 2). Finally, zinc finger 8 (along with the C terminus) is dispensable for, and may possibly destabilize, DNA binding mediated by zinc fingers 1–7 (Fig. 1). Taken together, these findings reveal the ZBRK1 zinc fingers to compose at least two functional classes: those that make minimal essential contacts with DNA (fingers 1–4) and those that modulate the stability of these contacts (fingers 5–8). Importantly, zinc fingers 5–8 that modulate ZBRK1 DNA binding activity also represent critical determinants of repression by DNA-bound ZBRK1 through association with co-repressors, including BRCA1. These findings thus extend the established role of KRAB-zinc fingers to include protein-protein interactions critical for transcriptional repression, and also identify within ZBRK1 dual specificity zinc fingers with twin roles in DNA-binding and transcriptional repression.

Our work advances the understanding of DNA recognition by KRAB-ZFPs in several respects. First, we provide further empirical evidence to support predictive models for C_2H_2 zinc finger-DNA recognition. Structural studies of 3- and 5-fingered proteins in complex with DNA have indicated that individual zinc fingers bind to ~3 bp of DNA (52–54). Based on this model, five of the eight ZBRK1 zinc fingers would be predicted to bind to its 15-bp recognition sequence. In fact, DNA-binding analyses revealed that under mild conditions of ionic strength, the first four ZBRK1 zinc fingers are sufficient to confer stable binding to its consensus sequence. However, more stringent conditions unmasked a requirement for the fifth finger, consistent with the aforementioned structural models. Second, our identification of ZBRK1 zinc finger 5 as a critical and context-

dependent DNA-binding determinant could clarify recent issues concerning selectivity among KRAB-ZFPs that recognize overlapping DNA-binding site sequences. In this regard, a four-fingered KRAB-ZFP called SZF1 was recently shown to recognize a DNA-binding site in common with ZBRK1 (64). The observation that SZF1 and ZBRK1 exhibit overlapping DNA-binding specificities *in vitro* raises the possibility that these proteins might compete for a common binding site(s) *in vivo* (64). This, in turn, could have significant implications for the biological regulation of target gene transcription by each of these proteins. However, as we show here, ZBRK1 zinc finger 5 is a critical DNA-binding determinant under more stringent conditions of increased ionic strength and also increased nonspecific competitor concentrations *in vitro* (Fig. 1 and data not shown). Because these conditions are more likely to approximate those of the cellular milieu, in which target site location must be achieved in the presence of a vast excess of like and unlike DNA sequences, ZBRK1 zinc finger 5 could represent a critical determinant of target site selection *in vivo*. Beyond zinc finger 5, zinc fingers 6 and 7 through enhanced affinity and/or protein-protein interactions could further influence ZBRK1 target site selectivity.

Our identification within ZBRK1 of a C-terminal BRCA1-dependent repression domain in addition to the N-terminal KRAB domain represents the first demonstration of a KRAB-ZFP harboring two independent repression domains. More importantly, the presence of two inherent repression domains could have important implications for gene-specific transcription control by ZBRK1. As we show here, the KRAB and C-terminal repression domains within ZBRK1 can be distinguished functionally on the basis of their respective requirements for BRCA1; the C-terminal repression domain is BRCA1-dependent, whereas the KRAB domain is not. This functional distinction may in part underlie the unique promoter specificities of the two repression domains. Whereas the KRAB repression domain exhibits broad promoter specificity, the BRCA1-dependent repression domain exhibits a more restricted promoter bias. Thus, the relative contribution of the BRCA1-dependent repression domain to overall ZBRK1 repression may vary among different ZBRK1 target promoters, effectively expanding the regulatory potential available at ZBRK1 target genes. It will be of interest in future studies to determine whether and how these discrete repression domains function synergistically to confer ZBRK1 repression.

Finally, although our work suggests that BRCA1 mediates repression by DNA-bound ZBRK1, we cannot exclude the additional possibility that BRCA1 also mediates ZBRK1 repression, at least in part, by modulating it sequence-specific DNA binding activity. Our observation that the BRCA1-binding surface on ZBRK1 includes zinc fingers that modulate its DNA binding activity in both a positive (zinc finger 7) and negative (zinc finger 8) manner is consistent with this possibility, and studies are currently underway to address this important issue. Nonetheless the studies presented here shed new light on the functional organization of ZBRK1 as a model KRAB-ZFP and further define the role of BRCA1 in sequence-specific transcription control.

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